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1.0 Introduction

1.1 Objective

The objective of this validation study is to assess the accuracy and reliability of the BG1Luc4E2 Estrogen Receptor (ER) Transcriptional Activation (TA) test method (hereafter referred to as BG1Luc ER TA) for the qualitative detection of substances with *in vitro* ER agonist or antagonist activity.

1.2 Public Health Perspective

Endocrine disruptors (EDs) are defined as substances that interfere with the normal function of hormones in the endocrine system, which can lead to abnormal growth, development, or reproduction (Ankley et al. 1998; Baker 2001; Brown et al. 2001; Combes 2000; EPA 1998; Fenner-Crisp and Fisher 1997; Greim 2004; Kavlock 1999). EDs are widespread in our environment and include both synthetic (for example, pesticides, pharmaceuticals, industrial chemicals) and naturally occurring (for example, plant products known as "phytoestrogens") substances. Public health concerns are due to a number of studies indicating that animal populations exposed to high levels of these substances have an increased incidence of reproductive and developmental abnormalities (Colborn et al. 1994; Guillette and Gunderson 2001; Segner 2005; Soin and Smagghe 2007; Tyler et al. 1998). Exposure of humans to EDs are also linked to adverse health outcomes such as altered reproduction and immune function, increased incidence of cancer, and an increased incidence of obesity and associated complications such as cardiovascular disease and type-2 diabetes (Kavlock et al. 2006; Rozman et al. 2006; Tsai 2006; Whitten et al. 1995; Whitten and Naftolin 1992, 1998; Whitten and Patisaul 2001; Whitten et al. 1992). In light of the growing concern surrounding this important issue, the accurate and timely identification of potential endocrine disruptors by the BG1Luc ER TA is an important aspect of protecting public health.

1.3 Historical Background

The Federal Food Drug and Cosmetic Act, the Food Quality Protection Act, and the Safe Drinking Water Act require the U.S. Environmental Protection Agency (EPA) to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate" [21 U.S.C. 346a(p)(1)]. Subsequent to passage of the Act, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that was charged by the EPA to provide recommendations on how to implement its Endocrine Disruptor Screening Program (EDSP).

The EPA accepted the EDSTAC's recommendations for a two-tier screening program as proposed in a *Federal Register* Notice in (EPA 1998). The purpose of Tier 1, which consists of *in vivo* and *in vitro* test methods, is to identify the potential of chemicals to interact with the estrogen, androgen, or thyroid hormonal systems. A negative result in Tier 1 is sufficient to put a chemical aside as having minimal potential to cause endocrine disruption, whereas a positive result necessitates further testing using *in vivo* methods in Tier 2. The purpose of Tier 2 is to more definitively identify and characterize the potential hazard to the endocrine system. Results from Tier 2 testing can also be used in a risk assessment. The EDSP is described in detail at http://www.epa.gov/scipoly/oscpendo/.

In April 2000, EPA nominated four types of *in vitro* test methods for detecting substances with potential endocrine disrupting activity; *in vitro* ER and AR binding and ER and AR TA test methods (EPA 2001; NIEHS 2001) for review by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICCVAM subsequently recommended that these methods should undergo independent scientific peer review based on their potential interagency applicability and public health significance. In response, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) compiled four separate comprehensive background review documents (BRDs) that included all available information on each of the four types of test methods (ICCVAM 2002a, b, c, d). In collaboration with ICCVAM and the ICCVAM Endocrine Disruptor Working Group (EDWG), NICEATM organized an independent international peer review panel (Panel) meeting to assess the suitability of the 137 available *in vitro* test methods identified in the BRD. The Panel reviewed the information and draft ICCVAM recommendations and concluded that there were no adequately validated *in vitro* ER- or AR-based test methods. ICCVAM considered the Panel's conclusions and recommendations, which are detailed in their Report, along with all comments received (ICCVAM 2002e)[1], and published test method recommendations for minimum essential test method components along with a list of 78 reference substances (ICCVAM Reference Substances) that should be used to standardize and validate *in vitro* ER and AR binding and TA test methods (ICCVAM 2003a, 2006). Based on the lack of adequately validated test methods, coupled with the public health issues identified above, ICCVAM and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) recommended the validation of *in vitro* endocrine disruptor screening methods as a high priority activity (NIEHS 2004).

[1] Text of comments available http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm?ftitle=02-26733

1.4 Nomination and Pre-screen Evaluation of the BG1Luc4E2 ER TA Test Method

In January 2004, Xenobiotics Detection Systems, Inc. (XDS, Durham, NC) nominated their LUMI-CELL® BG1Luc ER TA Test Method for an interlaboratory validation study (**Annex A)**. This method uses BG-1 cells, a human ovarian carcinoma cell line that was stably transfected with an estrogen-responsive luciferase reporter gene to measure whether and to what extent a substance induces or inhibits TA activity via ER mediated pathways (Denison and Heath-Pagliuso 1998). Included in the nomination package were test results from XDS for 56 of the 78 ICCVAM Reference Substances for agonist activity and 16 of the 78 ICCVAM Reference Substances for antagonist activity. These studies were funded primarily by a Small Business Innovation Research (SBIR) grant (SBIR43ES010533-01) from the National Institute of Environmental Health Sciences (NIEHS).

In accordance with the ICCVAM nomination process, NICEATM conducted a pre-screen evaluation of the nomination package (**Annex B**) to determine the extent to which it addressed the ICCVAM prioritization criteria (**Section 1.5**) and adherence to the ICCVAM recommendations for the standardization and validation of *in vitro* endocrine disruptor test methods (ICCVAM 2003a). Based on this evaluation, ICCVAM recommended that:

- The BG1Luc ER TA should be considered a high priority for interlaboratory validation studies as an in vitro test method for the detection of test substances with ER agonist and antagonist activity.
- Validation studies should include coordination and collaboration with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) and include one laboratory in each of the three respective geographic regions (US, Europe, Japan).
- In preparation for the interlaboratory validation study, XDS should conduct protocol standardization studies with an emphasis on filling data gaps in the antagonist protocol for the BG1Luc ER TA.

The NIEHS subsequently agreed to support the validation study in light of its participation as one of the three National Toxicology Program agencies, whose mission includes the development and validation of improved testing methods.

1.5 Basis for High Priority for Validation Studies

NICEATM provides preliminary evaluations of all test method submissions and nominations and summarizes the extent to which five ICCVAM prioritization criteria (ICCVAM 2003b) are met. As noted in **Section 1.4**, ICCVAM assigned a high priority to conducting an interlaboratory validation study for the

BG1Luc ER TA. This section details the rationale for this prioritization, as well as a summarization of more recent national and international developments that further emphasize the need to develop and validate *in vitro* ER TA test methods like the BG1Luc ER TA are discussed below.

1.5.1 Criterion 1. The extent to which the test method is (a) applicable to multiple agencies/programs and testing needs.

The EPA EDSP Tier 1 screening battery currently includes an ER TA test method, *OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line (HeLa-9903))*. The screening guideline also makes provisions for the use of other scientifically valid methods. Therefore, the BG1Luc ER TA may be applicable for addressing the ER TA component of the EPA EDSP Tier 1 screening battery.

The NIEHS has made a substantial investment in research focusing on endocrine disruptors over the past decade. The National Toxicology Program (NTP), headquartered at NIEHS, conducted the major health review of bisphenol A (BPA) that prompted both widespread reconsideration of its use by industry and the introduction of alternative products such as the BPA-free water bottle, among others. Endocrine disruption continues to be a focal point in NIEHS studies of commercial products that are in wide use, such as flame-retardants and pesticides.

The high throughput evaluation of chemicals is an important aspect many research and testing programs within government, academia, and industry. The BG1Luc ER TA is currently being evaluated by the NCGC for its adaptability to a high-throughput screening format, which could be used to support high throughput screening and testing programs.

In response to requests by the U.S. House of Representatives and Senate Appropriations Committees, NICEATM and ICCVAM published a Five-Year Plan to: 1) Research, develop, translate, and validate new and revised non-animal and other alternative assays for integration of relevant and reliable methods into Federal agency testing programs, and 2) Identify areas of high priority for new and revised non-animal and alternative assays or batteries of those assays to create a path forward for the replacement, reduction, and refinement of animal tests, when this is scientifically valid and appropriate (ICCVAM 2008; Poland et al. 2008; Stokes 2009). The evaluation of test methods for identifying endocrine disrupting chemicals was identified as one of the priority activities for ICCVAM-NICEATM in this plan.

The Organisation for Economic Co-Operation and Development (OECD) has also made a substantial investment in research focusing on endocrine disruptors. In June 2002, the OECD Task force on

Endocrine Disrupter Testing and Assessment (EDTA) developed a Conceptual Framework[2] for the testing and assessment of potential endocrine disrupting substances (Gelbke et al. 2004; Hass et al. 2004). Several international efforts are currently being undertaken which include using weight of evidence approaches to asses the endocrine disrupting potential of commercial chemicals, as described in the Conceptual Framework. Prominent examples are the EU Registration, Evaluation, Authorization, and Restriction of Chemicals [REACH] program, the European Economic Community (EEC) Cosmetic Directive, the EEC Plant Protection Products Regulation Directive, and the Japanese Extended Tasks on Endocrine Disruption [EXTEND 2010] program. The BG1Luc ER TA could be used as part of a weight of evidence approach in such programs.

It should be noted that individual agencies and programs must sanction the adoption of any test method, and any discussion of the potential applicability of the BG1Luc ER TA in this BRD does not imply acceptance or adoption by any agency or program.

1.5.2 Criterion 2. Warranted, based on the extent of expected use or application and impact on human, animal, or ecological health.

EDs encompass a variety of chemical classes including drugs (i.e., diethylstilbesterol), natural chemicals (i.e., genistein), and industrial chemicals (i.e., bisphenol a). Because of their ubiquitous uses, EDs are widespread in the environment. The association of exposure to EDs and adverse health effects in human and wildlife populations has led to worldwide concern. Some of the health effects that have led to this concern include global increases in endometriosis and hormone responsive cancers (for example, testicular and breast cancers), regional declines in sperm counts, increased prevalence of obesity, alterations to the onset of puberty, and increases in altered sex ratios in wildlife populations that are expected to result from exposure to chemicals that adversely affect steroid hormone action (Latendresse et al. 2009; Newbold 2008, 2010; Newbold et al. 2009; Newbold et al. 2008; vom Saal et al. 2007; WHO/PCS/EDC 2002). An appropriate screen such as BG1Luc ER TA can limit human and ecological exposure to EDs by identifying which chemicals are potential endocrine disruptors. Knowledge of these potential effects can result in a reduction of usage, and therefore, a decrease in the prevalence of reproductive and developmental issues caused by chemicals. There are several national and international programs aimed at indentifying chemicals with endocrine disrupting potential (**Section 1.5.1)** and the BG1Luc ER TA may be applicable to these programs

1.5.3 Criterion 3. The potential for the test method, compared to current test methods

[2] A copy of the conceptual framework is available from the OECD website http://www.oecd.org/document/58/0,3343,en_2649_34377_2348794_1_1_1_1,00.html.

accepted by regulatory agencies, to refine, reduce, or replace animal use.

No direct refinement, reduction, or replacement of animal use occurs when compared to the current *in vitro* OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903]). There are currently three *in vivo* methods commonly used by regulators to assess the estrogenic potential of substances: rat uterotrophic, rat pubertal female, and fish short-term reproduction assay. In addition, the "*in vitro*" Rat Uterine Cytosol ER binding assay also requires the use of animals as a source of ER. Although the BG1Luc ER TA will not directly replace any of these existing methods, it could be incorporated as part of a weight of evidence approach to reduce or eliminate the need for testing in these animal models.

1.5.4 Criterion 4. The potential for the proposed test method to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies.

When the BG1Luc ER TA validation study was initiated, there were no *in vitro* ER TA test methods that were considered adequately valid for regulatory use. Today, there is only one *in vitro* ER TA test method accepted by national and international agencies as adequately validated; the OECD Stably Transfected Human Estrogen Receptor-α Transcriptional Activation (STTA) Assay for the Detection of Estrogenic Agonist-Activity, described in OECD Chemicals Test Guideline (TG) 455 (OECD 2009). This method has been adopted by the US EPA as part of the EDSP Tier 1 Screening battery as *OPPTS 890.1300: Estrogen Receptor Transcriptional Activation (Human Cell Line [HeLa-9903])* (EPA 2009).

The ER TA method contained within TG 455 utilizes HeLa-9903 cells, a human cervical carcinoma cell line, in which human ERα and a reporter gene have been stably transfected. HeLa-9903 cells do not express endogenous ERα or ERβ. The BG1Luc ER TA may provide improved prediction of adverse health effects in humans because it uses a human cell line (BG-1) that endogenously expresses both human ERα and ERβ (Park et al. 2009; Pujol et al. 1998; Rogers and Denison 2000; Zhou et al. 2005) cofactors which may not be present in cells which do not express ER (Marsaud et al. 2003; Shang et al. 2000; Webb et al. 1995). The biological significance of two ER subtypes is still being elucidated, but there is mounting evidence for a role of ERβ in a number of normal and abnormal physiologic processes (Brown et al. 2009; Harris 2007; Hayashi et al. 2003; Skliris et al. 2008; Weiser et al. 2008). Although there are presently no known naturally occurring ERβ-specific substances, it is known that a number of substance types (for example isoflavones) are ERβ-selective (Escande et al. 2006; Mohler et al. 2010), with more potent responses through ERβ than ERα (Kuiper et al. 1998). The BG1Luc ER TA, using cells

that express both ERα and ERβ , allows for the potential detection of a wider range of substances than test methods that use cells expressing only the ERα receptor.

The BG1Luc ER TA also differs from TG 455 in its ability to identify substances possessing ER antagonist activity. This is important because estrogen receptor antagonists have a number of potential clinical uses, such as the treatment of osteoporosis and breast cancers (Jordan 2003). In addition, there is concern that any environmental anti-estrogens could have a detrimental influence on development and reproductive capacity of wildlife (Chamness et al. 1979; Fry and Toone 1981; Jones and Hajek 1995; Morris et al. 1967).

1.5.5 Criterion 5. The extent to which the test method provides other advantages (for example, reduced cost and time to perform) compared to current methods.

The BG1Luc ER TA is a rapid *in vitro* method that can identify ER agonists and antagonists within approximately four days at a cost of a few thousand dollars per substance (**Section 10.3**). The test method also provides concentration-response activity and information on the relative potency of a substance to a reference estrogen or anti-estrogen. *In vivo* methods require 30-60 days for completion and may cost many thousands of dollars (**Section 10.3**) in addition to the ethical concerns raised by the use of animals. The OECD TG 455 test method provides a concentration response and relative potency of a substance to a reference estrogen only. The uterotrophic assay provides a concentration response but is not generally used for determining relative potency.

1.6 BG1Luc ER TA Test Method Protocol Standardization Study

As a result of the high prioritization for validation studies, NICEATM initiated and managed the ICCVAM recommended study to standardize the BG1Luc ER TA test method protocols. These include essential test method components for ER TA test methods recommended in the ICCVAM recommendations (ICCVAM 2003a) were incorporated into the protocols. The ICCVAM recommended essential test method components that were incorporated into the protocol standardization included:

- Reference estrogen and associated TA response
- Preparation of test substances and the volume of the administered solvent
- Concentration range of test substances that should be tested
- Solvent and positive controls
- Number of within-test replicates
- Methods for data analysis
- Experiment acceptance criteria
- Interpretation of results

Intralaboratory reproducibility and accuracy of the standardized protocols were also evaluated by testing a representative subset of the ICCVAM Reference Substances. Results of the protocol standardization study are provided in **Annex C**.

1.7 The Interlaboratory BG1Luc ER TA Validation Study

NICEATM, which carries out independent validation studies relevant to the NTP mission, led and coordinated the international validation study with its counterparts in Japan (JaCVAM) and Europe (ECVAM). NICEATM organized a validation Study Management Team (SMT) in 2009 to oversee the scientific aspects of the validation study (**Table 1**) It also directly coordinated the day-to-day activities with the assistance of the NICEATM support contractor. A representative from the recently established Korean Center for the Validation of Alternative Methods (KoCVAM) was added to the SMT in 2010.

The BG1Luc ER TA was evaluated using laboratories in the U.S. (XDS), Europe (ECVAM), and Japan (Hiyoshi Corporation [Hiyoshi]). The study proceeded in four phases (**Figure 1-1**), during which the 78 ICCVAM Recommended Substances were tested (**Section 3.0**). Throughout the study, the SMT and NICEATM interacted to:

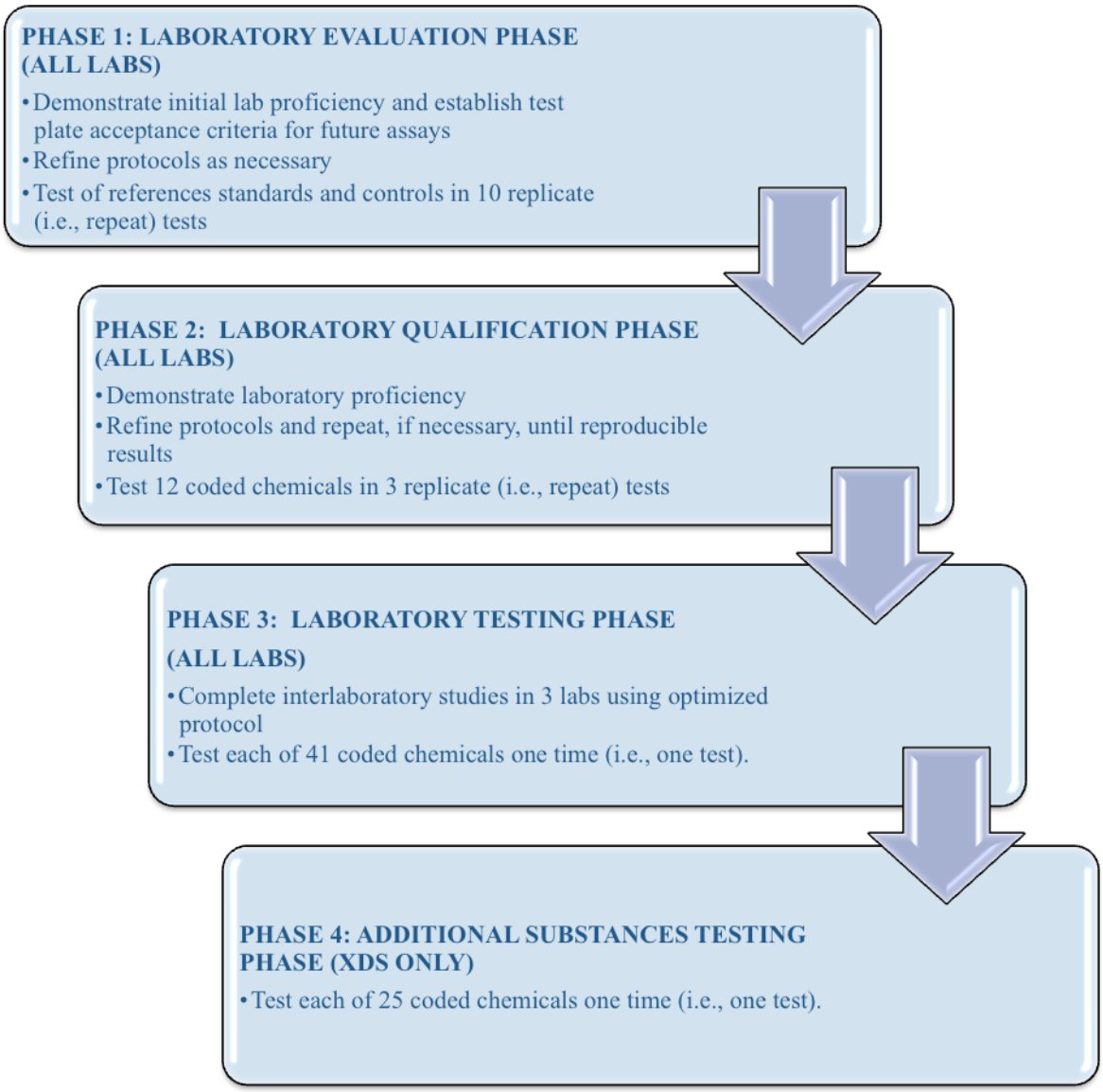
- Ensure that the study adheres to the principles stated in OECD Guidance Document Number 34 for prospective validation studies (OECD 2005)
- Develop a Statement of Work for the laboratories
- Determine timelines and deliverables
- Arrange for purchasing, coding, and distributing test substances to the laboratories
- Collect data from the laboratories and initiate statistical analyses
- Evaluate the reproducibility of results at each phase and refine the protocols, if necessary, before proceeding to the next phase
- Guide the study to conclusion and prepare documentation of the study.

Table 1 Study Management Team for the BG1Luc ER TA Validation Study

Study Management Team Member	Affiliation
Dr. William Stokes	NIEHS/NICEATM
Dr. Warren Casey	NIEHS/NICEATM
Dr. Susanne Bremer	ECVAM
Dr. Elise Grignard	ECVAM
Dr. Hajime Kojima	JaCVAM
Dr. Atsushi Ono	JaCVAM
Dr. Soon Young Han	KoCVAM
Dr. David Allen	ILS/NICEATM
Ms. Patricia Ceger	ILS/NICEATM
Mr. Frank Deal	ILS/NICEATM

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; ILS = Integrated Laboratory Systems (contract support staff for NICEATM); JaCVAM = Japanese Center for the Evaluation of Alternative Methods; KoCVAM = Korean Center for the Validation of Alternative Methods; NICEATM = NTP Interagency Center for the Evaluation of Alternative Toxicological Methods; NIEHS = National Institute of Environmental Health Sciences.

Figure 1-1 NICEATM/ECVAM/JaCVAM Validation Study Phases



1.8 Scientific Basis for the BG1Luc ER TA

The scientific basis of ER TA assays has been reviewed previously (ICCVAM 2002a; OECD 2002; Huet 2000). Briefly, *in vitro* ER TA assays are designed to identify agonist or antagonist substances that might interfere with normal estrogen activity *in vivo*. Unlike receptor binding assays, TA assays can distinguish between agonist and antagonist activity. *In vitro* ER TA assays that are used to evaluate agonist activity are generally performed by quantifying the induction of a reporter gene product in response to activation of the ER by the test substance. *In vitro* ER TA assays that evaluate antagonist activity measure the ability of a test substance to inhibit the induction of the reporter gene product by a reference estrogenic substance.

The interaction of estrogens with the ER in a cell initiates a cascade of events and a number of endpoints can be used to measure endocrine activity at the cellular level, including receptor binding, cellular proliferation, and TA. Upon ligand binding, the ER undergoes a conformational change that allows dissociation of co-repressor proteins and the recruitment of co-activator proteins. This ligand-bound ER complex dimerizes and binds to an estrogen responsive element (ERE) located upstream of genes under estrogen control. Binding alters the transcription of estrogen-controlled genes, which leads to the initiation or inhibition of cellular processes, including those necessary for cell proliferation, normal fetal development, and adult homeostasis. TA assays have an advantage over binding assays because they measure the biological response to receptor binding (that is, RNA transcription and translation), and thus, unlike binding assays, can distinguish between an agonist and an antagonist. In the BG1Luc ER TA, transcription of luciferase in response to estrogenic compounds is quantified using a luminometer.

1.9 Range of Substances Amenable to the BG1Luc ER TA

The BG1Luc ER TA can be applied to a wide range of substances, provided they can be dissolved in DMSO and are not toxic to BG1Luc4E2 cells at concentrations of 10μM or less. Although other solvents may be used for this test method, DMSO was the solvent of choice for this validation study. This method may be applicable to chemical mixtures. No mixtures, however, were evaluated in this validation study. Volatile substances may yield acceptable results if CO_2 permeable plastic film is used to seal the test plates. No volatile substances were evaluated in this validation study. Substances with endogenous luminescence (Evans and Diepenhorst 1926), or which naturally inhibit luciferase activity cannot be used in this luciferase-based test method.

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